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Molecular mechanisms leading to three different phenotypes in the cblD defect of intracellular cobalamin metabolism

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ABSTRACT

The cblD defect of intracellular vitamin B_{12} metabolism can lead to isolated methylmalonic aciduria (cblD-MMA) or homocystinuria (cblD-HC), or combined methylmalonic aciduria and homocystinuria (cblD-MMA/HC). We studied the mechanism whereby *MMADHC* mutations can lead to three phenotypes. The effect of various expression vectors containing *MMADHC* modified to contain an enhanced mitochondrial leader sequence or mutations changing possible downstream sites of reinitiation of translation or mutations introducing stop codons on rescue of adenosyl- and methylcobalamin formation was studied. The constructs were transfected into cell lines derived from various cblD patient's fibroblasts.

Expression of 10 mutant alleles from 15 cblD patients confirmed that the nature and location of the mutations correlate with the biochemical phenotype. In cblD-MMA/HC cells, improving mitochondrial targeting of MMADHC clearly increased the formation of adenosylcobalamin with a concomitant decrease in methylcobalamin formation. In cblD-MMA cells, this effect was dependent on the mutation and showed a negative correlation with endogenous *MMADHC* mRNA levels. These findings support the hypothesis that a single protein exists with two different functional domains that interact with either cytosolic or mitochondrial targets. Also a delicate balance exists between cytosolic methylcobalamin and mitochondrial adenosylcobalamin synthesis, supporting the role of cblD protein as a branch point in intracellular cobalamin trafficking. Furthermore our data indicate that the sequence after Met116 is sufficient for MeCbl synthesis whereas the additional sequence between Met62 and Met116 is required for AdoCbl synthesis. Accordingly Western blot studies reveal proteins of the size expected from the stop codon position with subsequent reinitiation of translation.

INTRODUCTION

In mammals vitamin B_{12} (cobalamin) is essential for normal development and survival and must be provided in the diet from animal products or from supplements. It is converted intracellularly to two active coenzyme forms, adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl). AdoCbl is the coenzyme for mitochondrial methylmalonyl-CoA mutase (MCM) which converts L-methylmalonyl-CoA to succinyl-CoA and is involved in catabolism of odd-chain fatty acids and some amino acids. Deficiency of MCM (MIM 609058) leads to accumulation of methylmalonic acid in body fluids (1) and results in a severe clinical phenotype. MeCbl is the coenzyme for cytosolic methionine synthase (MS) which converts homocysteine to methionine and is essential for normal one-carbon metabolism (2). Deficiency of MS (MIM 156570) causes increased levels of homocysteine in body fluids and a severe neurological phenotype.

Vitamin B_{12} has to be converted to its active cofactors in a series of intracellular reactions defined as eight different complementation classes (cblA, cblB, cblC, cblD composed of the three subgroups cblD-MMA, cblD-HC and cblD-MMA/HC, cblE, cblF, cblG, and mut) (3-5). The cblC, cblD-MMA/HC and cblF disorders cause combined methylmalonic aciduria and homocystinuria (MMA/HC), cblA, cblB, cblD-MMA and mut cause isolated MMA, and cblD-HC, cblE, and cblG result in isolated HC.

We previously reported that the cblD defect is associated with three distinct biochemical phenotypes: deficient synthesis of either both cobalamin coenzymes or only MeCbl or AdoCbl (5). Using microcell mediated chromosome transfer and refined genetic mapping we identified the gene responsible for cblD as *MMADHC* (methylmalonic aciduria, cblD type, and homocystinuria) (3). Individuals with cblD-MMA carry a mutation causing a premature stop of translation toward the N-terminal part of the protein on at least one allele, and patients with cblD-HC carry missense mutations causing amino acid substitutions toward the C-terminal part of the protein. The combined phenotype is associated with deleterious nonsense and missense mutations in the middle of the protein (3).

Clinical presentation is variable: cblD-MMA/HC patients presented with developmental delay, seizures, hypotonia, lethargy and megaloblastic anemia, cblD-HC patients with developmental delay,

ataxia and megaloblastic anemia and cblD-MMA patients had respiratory distress, cranial haemorrhage, seizures and an abnormal electroencephalogram (3, 5).

We have speculated that in patients with cblD-MMA there is reinitiation of translation whereby Met62 acts as second start codon. In patients with cblD-HC we showed that the missense mutations in the C-terminal part affect MeCbl formation but not the functional domain for the synthesis of AdoCbl (3).

In this study we have characterized mutations in further patients, performed transfection studies using different mutant constructs and investigated wildtype and mutant proteins by Western blot analysis in order to elucidate the molecular mechanisms of the three different phenotypes of cblD.

RESULTS

Mutations detected in cblD patients

Table 1 summarizes the phenotype, and nucleotide and protein changes in the 10 patients previously reported and 7 new ones (3, 6). Of a total of 15 mutations two, c.133dupG and c.229dupG have not been reported.

Expression studies of mutant alleles

The effect of mutant alleles on MeCbl and AdoCbl synthesis was studied in an immortalized combined cblD-MMA/HC cell line (D007) confirming the functional significance of various mutations (Supplementary Material Fig. S1). We show that mutant alleles associated with the cblD-HC phenotype (c.737A>G, p.D246G; c.746A>G, p.Y249C; c.776T>C, p.L259P) were unable to rescue MeCbl synthesis. In contrast, MeCbl synthesis was clearly rescued by mutant alleles associated with the cblD-MMA phenotype (c.57_64del, p.S20X; c.60insAT, p.L21IfsX2; c.133dupG, p.A45GfsX15; c.160C>T, p.R54X; c.228dupG, p.N77EfsX5). Regarding AdoCbl synthesis, mutant alleles associated with cblD-HC clearly rescued AdoCbl synthesis. The missense allele c.545C>A (p.T182N), found in a patient with cblD-HC compound heterozygous for the cblD-HC allele c.746A>G, rescued neither MeCbl nor AdoCbl synthesis. The nonsense allele c.683C>G (p.S228X), identified in homozygous state in a combined defect patient was used as a negative control.

Rescue of AdoCbl synthesis in cblD-MMA/HC and cblD-MMA cells

In previous studies we showed that AdoCbl synthesis was poorly corrected by transfection with the wildtype allele, except when using a vector in which a V5-polypeptide was attached to the C-terminus of the wildtype protein (3). We hypothesized that the low efficiency of rescue of AdoCbl synthesis by the wildtype construct is caused by inefficient mitochondrial targeting. Analysis of the amino acid sequence of MMADHC by the Mitoprot II software (7) predicted a cleavable, N-terminal mitochondrial leader sequence (MLS) of 11 amino acids (MANVLCNRARL).

To investigate the efficiency of the endogenous MLS we prepared a construct (cblD_MLS_ALDH2) in which this putative MLS (Met1-Leu11) was replaced by the MLS of aldehyde dehydrogenase 2 (ALDH2, NP_000681) which has been shown previously to efficiently target natural and synthetic polypeptides to the mitochondria (8). Transfection of this construct into a combined defect cell line (D007, p.F204_A232del/ p.F204_A232del) led to a dramatic increase of AdoCbl synthesis to high normal or elevated levels (Fig. 1), and a concomitant decrease of the level of rescue of MeCbl synthesis.

In contrast, in different cell lines from patients with isolated cblD-MMA, transfection with either cblD wildtype or even cblD_MLS_ALDH2 did not normalize of AdoCbl synthesis (Fig. 2), i.e. in 4 cblD-MMA cell lines AdoCbl synthesis increased after transfection with cblD_MLS_ALDH2 from 1% of total cobalamins to only 2.0 – 3.8 % ($p=0.025$ in D003, p.S20X/pS20X; $p=0.146$ in D010, p.S20X/p.S20X, not shown; $p=0.020$ in D008, p.L21IfsX2/ p.C153MfsX10; $p=0.073$ in D012, p.A45GfsX15/p.A45GfsX15). In the other two patients the increase was more prominent from ≤1% to 10% ($p=0.004$ in D004, p.R54X/p.L103_S108dup; $p=0.001$ in D017, p.N77EfsX5/p.N77EfsX5) (Fig. 2). In contrast, in 3 cell lines with cblD-MMA/HC (D006, p.Y140X/ p.Y140X; D007, p.T204_A232del/ p.T204_A232del; D009, p.S228X/p.S228X) transfection with cblD_MLS_ALDH2 led to rescue of AdoCbl synthesis to normal levels. Finally, in control cells, transfection with cblD_MLS_ALDH2 resulted in a small but not significant ($p=0.139$) increase of AdoCbl synthesis (Fig. 2). Transfection had no substantial effect on the normal level of MeCbl synthesis in any of the cblD-MMA cell lines (not shown).

Attempts to increase the level of rescue of AdoCbl synthesis with the cblD wildtype allele in a cblD-MMA cell line homozygous for p.S20X (D003 or D010) by using another type of vector (pcDNA3) for transient transfection were not successful. When using stable transfection in a retroviral system (pBABE), again only a small but statistically significant (P=<0.001) increase of AdoCbl from 1.3% to 3.4% was seen (Supplementary Material, Fig. S2).

***MMADHC* mRNA expression**

Real-time PCR showed considerable differences in the levels of *MMADHC* mRNA in cblD-cell lines (Fig. 3a). Clearly reduced levels were obtained in a patient with the combined cblD-MMA/HC phenotype and homozygous for a premature termination codon (PTC) in exon 5 (D006, c.419dupA, p.Y140X), and to a lesser extent in a patient with an isolated cblD-MMA phenotype compound heterozygous for a PTC and an inframe duplication in exon 4 (D004, c.160C>T, p.R54X/ c.307_324dup, p.L103_S108dup). However, mRNA levels were not significantly reduced in other patients with the cblD-MMA phenotype and PTCs in exon 3 (D003 and D010 (not shown), homozygous for c.57_64del, p.S20X; D012, homozygous for c.133dupG, p.A45GfsX15); in exon 3 and 5 (D008, c.60insAT, p.L21IfsX2/c.455dupC, p.C153MfsX10) and in exon 4 (D017, homozygous for c.228dupG, p.N77EfsX5). Normal mRNA levels were also found in patients with the cblD-MMA/HC phenotype and homozygous for PTCs in exon 7 (D009, c.683C>G, p.S228X) or exon 8 (D005, c.748C>T, p.R250X), or an inframe skipping of exon 7 (D007, c.696+1_4delGTGA, p.T204_A232del). Suppression of nonsense mediated mRNA decay (NMD) using emetine rescued mRNA levels in the two patients with clearly reduced mRNA levels to normal and increased mRNA levels in all cell lines tested (Supplementary Material, Fig. S3). In all other cell lines the expression varied from 0.6 to 1.4 when compared to that of four control cell lines normalized to mRNA levels of GAPDH (not shown). Similar results were obtained when the mRNA levels were normalized to the mRNA of the mitochondrial protein MRPL19 (not shown).

A negative correlation was observed between the mean levels of expression of *MMADHC* mRNA and the level of rescue of AdoCbl synthesis in the different cblD-MMA cell lines. Thus the correlation coefficients between mRNA synthesis and AdoCbl rescue were R=0.89 and R=0.83 for transfection

with the wildtype and cblD_MLS_ALDH2 construct respectively. No such correlation was observed in cblD-MMA/HC cell lines.

MMADHC protein expression

Endogenous production of MMADHC protein was studied by immunoprecipitation in crude cell extracts. This showed a single band of 32.8 kDa corresponding to the full length cblD protein in control cells. In cells of patient D003 homozygous for p.Ser20X, smaller bands of 26.5 and 20.5 kDa corresponding to MMADHC proteins starting at Met62 and Met116, respectively, were detected (Fig 3b). In cells of patient D017, homozygous for p.N77EfsX5 only the 20.5 kDa band corresponding to MMADHC starting at Met116 was present. No MMADHC protein was detected in cell lines D006 and D007 that are homozygous for p.Y140X and p.F204_A232del, respectively.

Effect of mutations associated with cblD-MMA on the level of rescue of AdoCbl synthesis

The negative correlation observed between the level of the rescue of AdoCbl synthesis and the level of mRNA among the cblD-MMA cell lines suggests that the endogenous mutant protein in these cells may affect the ability of the wildtype protein to produce AdoCbl. We therefore studied the effect of five cblD-MMA associated mutations on rescue of AdoCbl synthesis by co-transfecting a combined cblD-MMA/HC cell line (D007, p.F204_A232del/ p.F204_A232del) with the wildtype cDNA containing an optimized MLS (cblD_MLS_ALDH2) together with each of five constructs carrying cblD-MMA associated mutations (Fig. 4). Co-transfection with empty vector, wildtype and a construct containing the nonsense mutation c.683C>G/p.S228X that is associated with the cblD-MMA/HC phenotype were used as controls. When compared to co-transfection of cblD_MLS_ALDH2 with empty vector, co-transfection with wildtype construct resulted in a significant decrease of AdoCbl synthesis from 19% of total cobalamins to 12 % ($p=<0.001$). AdoCbl synthesis after co-transfection with one of the cblD-MMA associated mutant alleles (c.160C>T, p.R.54X) decreased to 11% and did not differ from that with wildtype ($P=0.655$), whereas after co-transfection with four other cblD-MMA associated mutant alleles (c.57-64del, p.S20X; c.60insAT, p.L21IfsX2; c.133dupG, p.A45GfsX15; c.228dupG, p.N77EfsX5) the decrease was significantly lower than with wildtype, i.e. a decrease to

5.1 % (p=<0.001), 5.9 % (p=0.001), 8.2 % (p=0.008), and 5.6% (p=<0.001), respectively. Co-transfection with the null-allele p.S228X showed no effect (P=0.096). Similar results were obtained when transfecting cell line D006, homozygous for a truncating mutation in the middle of the protein (c.419dupA/p.Y140X) (not shown).

These findings are in accordance with the variable levels of mRNA and rescue of AdoCbl synthesis in the different cblD-MMA cell lines and suggest that these mutant proteins interfere with the wildtype protein by affecting AdoCbl synthesis to various degrees.

Effect of alternative start codons on synthesis of MeCbl and AdoCbl

All six patients with the cblD-MMA phenotype have at least one allele carrying a mutation that leads to premature termination of translation before the second or third methionine located at positions 62 and 116, respectively (Table 1). Four of these are homozygous and the other two are compound heterozygous and carry in addition i) a deletion that leads to a frameshift and premature termination at amino acid residue 162 (p.C153MfsX10 in D008), and ii) an in-frame duplication of the amino acids 103 to 108 (p.L103_S108dup in D004). Both mutations are not detectable at the cDNA level but clearly heterozygous at the genomic level suggesting that the steady state level of mRNA from these alleles is very low. Since MeCbl synthesis is completely normal in these patients we have speculated that there is reinitiation of translation at a downstream AUG codon, e.g. at Met62 and/or at Met116. Both codons are located within a sequence compatible with the Kozak consensus (3, 9).

To test this hypothesis we prepared constructs with selected changes in the *MMADHC* wildtype cDNA, transfected them into a cblD-MMA/HC cell line (D007, p.F204_A232del/ p.F204_A232del) and investigated the rescue of cobalamin coenzyme synthesis. As shown in Figure 5 N-terminal truncation to either Met62 (Fig. 5 construct 5) or Met116 (Fig. 5 construct 7), or introduction of a stop codon at position 89 (Fig. 5 construct 9), does not lead to lack of MeCbl synthesis. In contrast, introduction of a stop codon at position 118 (Fig. 5 construct 10) completely abolishes MeCbl synthesis. These findings confirm that translation can be initiated either at Met62 or at Met116, but not downstream of Met116.

Concerning AdoCbl synthesis, as expected there was no AdoCbl formation with truncation N-terminally to Met62 or Met116 as these constructs lack a MLS (Fig. 5). However, introduction of the MLS of ALDH2 N-terminally at Met62 (Fig.5 construct 6) resulted in significant rescue of AdoCbl synthesis whereas no rescue was observed when the MLS was added N-terminally to Met116 (Fig.5 construct 8). This indicates that the sequence between Met62 and Met116 and downstream is essential for mitochondrial AdoCbl synthesis. Thus the protein truncated N-terminally to Met116 has lost the dual function being unable to support AdoCbl synthesis but being fully active in MeCbl synthesis.

The removal of possible alternative translation start sites by mutating Met62 and Met116 to glutamine residues (10) (Fig.5 construct 3 and 4) did not significantly affect the synthesis of MeCbl ($p=0.294$) or AdoCbl ($p=0.640$). This suggests that initiation of translation at alternative start sites plays at most a minor role in the wildtype.

DISCUSSION

Our studies provide further evidence for the idea that the MMADHC protein contains various domains responsible for i) targeting the protein towards the mitochondria; ii) MeCbl synthesis and iii) AdoCbl synthesis. Accordingly, the nature and location of mutations within the protein determine one of three biochemical phenotypes, i.e. combined MMA/HC, isolated MMA or isolated HC.

To date, we have identified a total of 17 patients, 5 with a combined defect, and 6 each with isolated MMA or isolated HC (Table 1). In line with our idea, each patient with cblD-MMA carries on at least one allele a mutation causing a premature stop toward the N-terminal part of the protein. Importantly, one patient (D017) is homozygous for a duplication leading to a stop codon at position 81 (p.N77EfsX5) which is downstream from the second (Met62) but upstream from the third (Met116) initiation codon supporting the idea that reinitiation of translation can also occur at Met116. All six patients with cblD-HC carry at least one missense mutation causing amino acid substitutions toward the C-terminal part of the protein. Interestingly, these three mutations are located within a short stretch of 14 amino acids (p.D246G, p.Y249C, p.L259P). Using expression studies we have proven the functional significance of these mutations.

This small region is one of the five putative sites of interaction between MMADHC and MMACHC according to recent phage display experiments (11). Thus our data support the hypothesis that mutations in the C-terminal part of MMADHC could lead to a defect in the MeCbl pathway by preventing binding with MMACHC.

Further supporting our concept, 3 additional reported patients with cblD-MMA are homozygous for mutations leading to a premature stop codon N-terminal of Met62 (6). All five patients with the combined defect carry mutations that are predicted to lead to premature stop codons C-terminal of Met116.

Central to the various features of this special protein are reinitiation of translation at downstream start codons and both, cytosolic and mitochondrial localization. Our studies conclusively show that a truncated protein lacking the first 115 amino acids is fully capable of MeCbl synthesis. In support of reinitiation of translation and in line with naturally occurring mutations in patients we have shown that truncation N-terminally to Met62 or Met116, or introduction of a stop codon at position 89, still allow formation of MeCbl, while a stop codon after Met116 knocks out the formation of MeCbl (Fig. 5). In line with our concept western blot studies showed formation of protein of the sizes expected from the position of stop codons and subsequent reinitiation of translation (Fig. 3b).

Our studies show that AdoCbl synthesis requires targeting of the cblD protein to the mitochondria (positions 1-61) and an intact sequence downstream from Met62 (Fig. 5). More precise identification of the essential sequence for AdoCbl synthesis requires further studies. Dual localization of proteins has been shown to be caused by various mechanisms including alternative splicing, initiation codon scan-through, multiple in-frame start codons, incomplete secretion, reverse translocation, spontaneous folding and others (12-18). Furthermore proteins that are localized in both mitochondria and the cytosol have been shown to be more likely to have a weak MLS (19). Thus the nature of the MLS can shift the balance between mitochondrial and cytosolic localization (20). Moreover, our studies confirm that the balance between mitochondrial and cytosolic formation can be strongly influenced by the presence and nature of a MLS. Thus, replacing the putative endogenous MLS of MMADHC by the MLS of ALDH2, an established mitochondrial enzyme (21, 22), dramatically increased AdoCbl formation with a concomitant reduction of MeCbl formation (Fig. 1). Finally, the results obtained with

the construct in which possible alternative translation start sites had been removed in the wildtype by mutating Met62 and Met116 to glutamine residues demonstrate that production of shortened isoform is not essential for the processes leading to MeCbl or AdoCbl synthesis. Thus we conclude that in wildtype cells a single protein product fulfils the functions needed for synthesis of both coenzymes and this is supported by dual localization of MMADHC.

To our surprise AdoCbl formation could not be rescued in cblD-MMA cell lines to the same extent as in cblD-MMA/HC cell lines, even when using the cblD_MLS_ALDH2 construct (Fig. 2). In considering this lack of rescue we evaluated *MMADHC* mRNA expression and rescue of AdoCbl with the wildtype and CblD_MLS_ALDH2 construct in 5 cblD-MMA cell lines. We observed a negative correlation between the level of endogenous *MMADHC* mRNA (Fig. 3a) and the level of AdoCbl rescue (Fig. 2) suggesting that in these cell lines the level of endogenous mRNA and/or MMADHC protein may influence AdoCbl rescue. We speculate that if MMADHC is part of a complex necessary for the formation of AdoCbl, a shorter MMADHC protein produced in cblD-MMA cells due to reinitiation of translation at Met62 or Met116 could affect formation of functional complexes thereby reducing AdoCbl synthesis. Another possibility is that the short polypeptides that are produced in cblD-MMA patients (from Met1 to the stop codon resulting from the mutation) could interact with full length protein or with binding partners located in the cytosol or at the mitochondrial membrane. In support of these findings co-transfection of cblD-MMA/HC cells with the cblD_MLS_ALDH2 and various cblD-MMA mutant alleles also show variable rescue of AdoCbl synthesis (Fig. 4). Again the level of rescue appears to correlate with the level of endogenous mRNA (Fig. 3a).

Essentially normal amounts of mRNA were observed in patients D005 (p.R250X/p.R250X) and D009 (p.S228X/p.S228X). This can be explained by the well established rule that premature termination codons (PTCs) residing in the last or penultimate exon (that is – they are not followed by more than one exon-exon junction) generally do not elicit NMD (23). As expected severely reduced mRNA levels were found in the cblD-MMA/HC patient 006 homozygous for a PTC in exon 5 (p.Y140X/p.Y140X). Regarding the truncating mutations found in the cblD-MMA cell lines before the second (D003 and D010, D008, D0012, D004) or third (D017) start codon would all fulfill the rules of a PTC eliciting NMD, but instead have (near) normal levels of mRNA (Fig. 3). We can only speculate

about the immunity of these cblD-MMA cell lines to NMD. A possible explanation would be that translation reinitiation downstream of a PTC inhibits NMD (24) indicating that PTC recognition by translating ribosomes is not sufficient for NMD, and that NMD depends on events that are subsequent to translation termination and are precluded by reinitiation (23).

Interaction of MMADHC with MMACHC may play a role in the regulation of the balance between AdoCbl and MeCbl synthesis. Thus we observed that co-transfection of the cblC gene with cblD wildtype cDNA in cblD-MMA/HC or cblD-MMA cell lines doubled the level of AdoCbl synthesis with less effect on MeCbl synthesis, compared with cblD wildtype alone (unpublished results). This is supported by the finding that MMADHC acts as a binding partner for MMACHC both *in vitro* and *in vivo* (11).

In summary our findings suggest that under physiological conditions, there is a delicate balance of intracellular localization of MMADHC and that this balance is influenced by the efficacy of the mitochondrial leader sequence, supporting the concept that the cblD protein acts as a branch point in intracellular cobalamin trafficking. Our data indicate that the sequence after Met116 is sufficient for MeCbl synthesis whereas the sequence from Met62 downstream is required for AdoCbl synthesis. We propose the existence of one single protein with two different functional domains that interact with either cytosolic or mitochondrial targets. Further studies on interactions with possible partner proteins and determination of the 3-D structure of MMADHC and sub-cellular localization will help to further elucidate the role of this intriguing protein in intracellular cobalamin metabolism.

MATERIALS AND METHODS

Cell Culture

Fibroblasts were obtained for diagnostic purposes with informed consent from the patients or their parents and referring clinicians approved the use of the cells for our investigation of the origin of the disease. Fibroblasts were routinely grown in Earl's minimal essential medium (Amimed, Basel, Switzerland) supplemented with 10 % fetal bovine serum (Amimed) as described earlier (5). Fibroblasts were transformed by transfection with pRNS1 (25) using electroporation as previously described (3).

Mutation analysis

Genomic DNA and total RNA were extracted from cultured patient fibroblasts using the QIAamp DNA Mini Kit and RNeasy Kit (Qiagen, Hombrechtikon, Switzerland), respectively. For mutation analysis genomic DNA and cDNA synthesized from total RNA by reverse transcription (RT-PCR) were amplified by PCR using specific primers as described earlier (3). To confirm mutations identified in RT-PCR products, the corresponding exons were amplified by PCR from genomic DNA using flanking intronic primers and were sequenced by the ABI BigDye method (Applied Biosystems, Rotkreuz, Switzerland). All mutations were checked for their correct designation using "mutalyzer" (www.mutalyzer.nl) according to the guidelines of the Human Genome Variation Society.

Preparation of constructs for transfection

Constructs containing the *MMADHC* wildtype and mutant cDNA were prepared in mammalian expression vectors as described earlier (26). Shortly, cDNA was produced from total RNA by reverse transcription using the Titan One Tube RT-PCR Kit (Roche, Basel, Switzerland), cloned into pCR-Blunt-II-TOPO (Invitrogen, Basel, Switzerland), subcloned into pTracer-CMV2 (Invitrogen) at the *Eco*RI site, and propagated in *E.coli* DH5α competent cells (Invitrogen). Plasmids for transfections were isolated using the QIAfilter Plasmid Maxi Kit (Qiagen). Introduction of the sequence of base pairs corresponding to the MLS of ALDH2 (MLRAAARFGPRLGRRLLSAAATQA) (27) into wildtype *MMADHC* cDNA at different positions and production of truncated and mutant *MMADHC* cDNAs was achieved using site directed mutagenesis and appropriate oligonucleotides (Supplementary Material Table S1). All PCR reactions were carried out using Hot Gyro Polymerase (Solis Biodyne, Tartu, Estonia). PCR products were cloned into pTracer-CMV2, as described above. All constructs were sequenced to verify that they contained the indicated changes.

Transfection studies

Constructs containing wildtype and different mutant cDNA sequences in pTracer-CMV2 were transfected transiently into transformed patient and control fibroblasts by electroporation as described

earlier (3). Rescue of cellular function was tested by measuring the synthesis of MeCbl and/or AdoCbl from [^{57}Co]cyanocobalamin, as described previously (5).

Real time PCR

cDNA was synthesized from total RNA using the TaqMan Reverse Transcription Reagent (Applied Biosystems). Analysis of the abundance of the *MMADHC* mRNA was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's recommendations, except that the total reaction volume was reduced to 10 µl. The TaqMan probes (Applied Biosystems) used were Hs00739517_g1 (*MMADHC*) and as controls Hs00608519_m1 (*MRPL19*) and Hs99999905_m1 (*GAPDH*).

Western blot studies

Fibroblasts from control and CblD patients were lysed with RIPA buffer (Invitrogen, Basel, Switzerland), immunoprecipitated with proteinG-sepharose (Invitrogen) and a polyclonal mouse anti-human MMADHC antibody (Biogenes, Berlin, Germany). Proteins were separated by SDS-PAGE on a 12 % gel, transferred to polyvinylidene difluoride membranes, blocked at room temperature for 1 hour with 1 % BSA in PBS containing 0.1 % Tween 20, and incubated overnight with a monoclonal mouse anti-human MMADHC antibody (AMSBio, Lugano, Switzerland). Visualization of protein bands was performed using a donkey anti-rabbit antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch, Newmarket, UK) and SuperSignal reagent according to the manufacturer's instructions (Perbio, Lausanne, Switzerland). As protein molecular size marker we used Kaleidoscope Precision Plus (BioRad, Reinach, Switzerland).

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Conflict of Interest statement. Nothing to declare.

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LEGENDS TO FIGURES

Figure 1. Effect of optimized MLS on rescue of AdoCbl synthesis in a cblD-MMA/HC cell line. Transformed fibroblasts of a cblD-MMA/HC patient (D007, p.F204_A232del/ p.F204_A232del) were transfected with a construct (cblD_MLS_ALDH2) that replaces the 11 amino acids long putative mitochondrial leader sequence (MLS) of MMADHC by the MLS of aldehyde dehydrogenase 2 (ALDH2) and assayed for adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl) synthesis. Transfections with empty vector (vector only) and wildtype (wt) were used as controls. Constructs were prepared using pTracer vector and transfections were made by electroporation. Columns represent the mean and vertical lines the range of results from 7 replicate experiments with single

determinations. In a transformed control cell line transfected with an empty vector the mean synthesis of AdoCbl was 18 % (range 14 % – 24%, n=7) and that of MeCbl 51% (range 46 % – 61%).

Figure 2. Transfection studies of cblD-wt and MLS optimized cblD-wt in various patient cell lines. Adenosylcobalamin (AdoCbl) synthesis in transformed fibroblasts of five cblD-MMA patients (D003, p.S20X/p.S20X; D008, p.L21IfsX2/p.C153MfsX10; D012, p.A45GfsX15/p.A45GfsX15; D004, p.R54X/p.L103_S108dup; D017, p.N77EfsX5/ p.N77EfsX5), three cblD-MMA/HC patients (D006, p.Y140X/p.Y140X; D007, p.T204_A232del/p.T204_A232del; D009, p.S228X/p.S228X) and a control after transfection with *a MMADHC* wildtype (cblD-wt) construct and a construct (cblD_MLS_ALDH2) that replaces the endogenous 11 amino acids long putative mitochondrial leader sequence (MLS) of MMADHC with the MLS of aldehyde dehydrogenase 2 (ALDH2). Transfection with empty vector (vector only) was used as negative control. Patient D010 with the same genotype as patient D003 (p.S20X/p.S20X) gave similar results (not shown). All constructs were prepared using pTracer vector and transfections were made by electroporation. Columns represent the mean and vertical lines the range of results from at least 4 replicate experiments with single determinations.

Figure 3. MMADHC mRNA and protein expression in various mutant and control cell lines. Relative levels of *MMADHC* mRNA (**Fig. 3a**) in fibroblasts of 5 cblD-MMA patients (D003, c.57_64del/c.57_64del; D008, c.60insAT/c.455dupC; D012, c.133dupG/c.133dupG; D004, c,160C>T/c.307_324dup; D017, c.228dupG/c.228dupG) and 4 cblD-MMA/HC patients (D006, c.419dupA/c.419dupA; D009, c.683C>G/c.683C>G; D007, c.696+1_4del/ c.696+1_4del; D005 c.748C>T/c.748C>T) measured by quantitative real time PCR. Patient D010 with the same genotype as patient D003 (c.57_64del/c.57_64del) gave similar results (not shown). mRNA levels were compared to mean level obtained in 4 control cell lines and were normalized to mRNA level of GAPDH. Columns are the mean and vertical lines the range of triplicate determinations.

MMADHC protein expression (**Fig. 3b**) in fibroblasts of 2 representative cblD-MMA patients (D003, c.57_64del/c.57_64del; D017, c.228dupG/c.228dupG) and 2 cblD-MMA/HC patients (D006, c.419dupA/c.419dupA; D007, c.696+1_4del/c.696+1_4del). Cell extracts were immunoprecipitated

with a polyclonal anti-MMADHC antibody, separated by SDS-PAGE in a 12% polyacrylamide gel and visualized with a monoclonal anti-MMADHC antibody as described in Material and Methods.

Figure 4. Effect on AdoCbl synthesis of co-transfection of MLS optimized cblD-wt together with various mutant cblD-MMA alleles in a cblD-MMA/HC cell line. AdoCbl synthesis was measured in transformed fibroblasts of a cblD-MMA/HC patient (D007, p.F204_A232del/ p.F204_A232del) after co-transfection of a wildtype construct (cblD_MLS_ALDH2) in which the endogenous putative mitochondrial leader sequence (MLS) of *MMADHC* is replaced by the MLS of aldehyde dehydrogenase 2 (ALDH2) together with wildtype (wt) or cblD-MMA associated mutant alleles (p.S20X, p.L21IfsX2, p.A45GfsX15, p.R54X, p.N77EfsX5). Co-transfection together with empty vector (vector only) and with a cblD-MMA/HC associated mutant allele (p.S228X) were used as controls. All constructs were prepared using pTracer vector and transfections were made by electroporation. Columns represent the mean and vertical lines the range of results from at least 4 replicate experiments with single determinations.

Figure 5. Effect of alternative start codons on synthesis of methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl). To study possible initiation of translation from alternative AUG codons selected changes were introduced into the wildtype cDNA followed by transfection into immortalized fibroblasts of a cblD-MMA/HC patient (D007, p.F204_A232del/ p.F204_A232del) and assay of cobalamin coenzyme synthesis. The different constructs used are numbered from 1 to 10 and the predicted structures of the expressed proteins are schematically presented by vertical bars. Thus grey bars represent functionally active protein, black bars mitochondrial leader sequence of aldehyde dehydrogenase 2 (ALDH2_MLS) and white bars functionally inactive polypeptides. Transfection with empty vector (vector only) and wildtype (wt) were used as controls. Changes were introduced into wildtype cDNA by site directed mutagenesis as described in Materials and Methods. All constructs were prepared using pTracer vector and transfections were done by electroporation. Columns represent the mean and vertical lines the range of results from at least 4 replicate experiments with single determinations.

Table 1. Phenotype and *MMADHC* mutations detected in 17 patients from 16 families with cblD defect

		Allele 1			**Allele 2**			
Phenotype*	ID	Nucleotide change	Protein (predicted)	Location	Nucleotide change	Protein (predicted)	Location	Reference
MMA	D003	c.57_64delCTCTTTAG	p.S20X	Exon 3	c.57_64delCTCTTTAG	p.S20X	Exon 3	Pat 3 in Coelho et al 2008
MMA	D010	c.57_64delCTCTTTAG	p.S20X	Exon 3	c.57_64delCTCTTTAG	p.S20X	Exon 3	This study
MMA	D008	c.60insAT	p.L21IfsX2°	Exon 3	c.455dupC[#]	p.C153MfsX10	Exon 5	WG3280 in Miousse et al 2009
MMA	D012	c.133dupG	p.A45GfsX15	Exon 3	c.133dupG	p. A45GfsX15	Exon 3	This study
MMA	D004	c.160C>T	p.R54X°	Exon 4	c.307_324dup[#]	p.L103_S108dup	Exon 4	Pat 4 in Coelho et al 2008, WG1437 in Miousse et al 2009
MMA	D017	c.228dupG	p.N77EfsX5	Exon 4	c.228dupG	p.N77EfsX5	Exon 4	This study
HC	D011	c.737A>G	p.D246G	Exon 8	c.737A>G	p.D246G	Exon 8	WG3745 in Miousse et al 2009
HC	D015	c.737A>G	p.D246G	Exon 8	c.737A>G	p.D246G	Exon 8	This study
HC	D013[$]	c.746A>G	p.Y249C	Exon 8	c.746A>G	p.Y249C	Exon 8	This study
HC	D014[$]	c.746A>G	p.Y249C	Exon 8	c.746A>G	p.Y249C	Exon 8	This study
HC	D002	c.746A>G	p.Y249C	Exon 6	c.545C>A	p.T182N	Exon 8	Pat 2 in Coelho et al 2008
HC	D001	c.776T>C	p.L259P	Exon 8	c.776T>C	p.L259P	Exon 8	Pat. 1 in Coelho et al 2008
MMA/HC	D006	c.419dupA	p.Y140X	Exon 5	c.419dupA	p.Y140X	Exon 5	Pat 6 in Coelho et al 2008
MMA/HC	D009	c.683C>G	p.S228X	Exon 7	c.683C>G	p.S228X	Exon 7	WG3583 in Miousse et al 2009
MMA/HC	D007	c.696+1_4delGTGA	p.F204_A232del (skip exon 7)	Intron 7	c.696+1_4delGTGA	p.F204_A232del (skip exon 7)	Intron 7	Pat 7 in Coelho et al 2008
MMA/HC	D005	c.748C>T	p.R250X	Exon 8	c.748C>T	p.R250X	Exon 8	Pat 5 in Coelho et al 2008
MMA/HC	D016	c.748C>T	p.R250X	Exon 8	c.748C>T	p.R250X	Exon 8	This study

* MMA = isolated methylmalonic aciduria ; HC= isolated homocystinuria , MMA/HC = combined methylmalonic aciduria and homocystinuria

° appears homozygous at cDNA level; clearly heterozygous at genomic level

[#] not detectable at cDNA level suggesting low steady state levels of mRNA; clearly heterozygous at genomic level

[$] siblings

ABBREVIATIONS

MMA: methylmalonic aciduria

HC: homocystinuria

MMA/HC: combined methylmalonic aciduria and homocystinuria

AdoCbl: adenosylcobalamin

MeCbl: methylcobalamin

MCM: methylmalonyl-CoA mutase

MS: methionine synthase

MLS: mitochondrial leader sequence

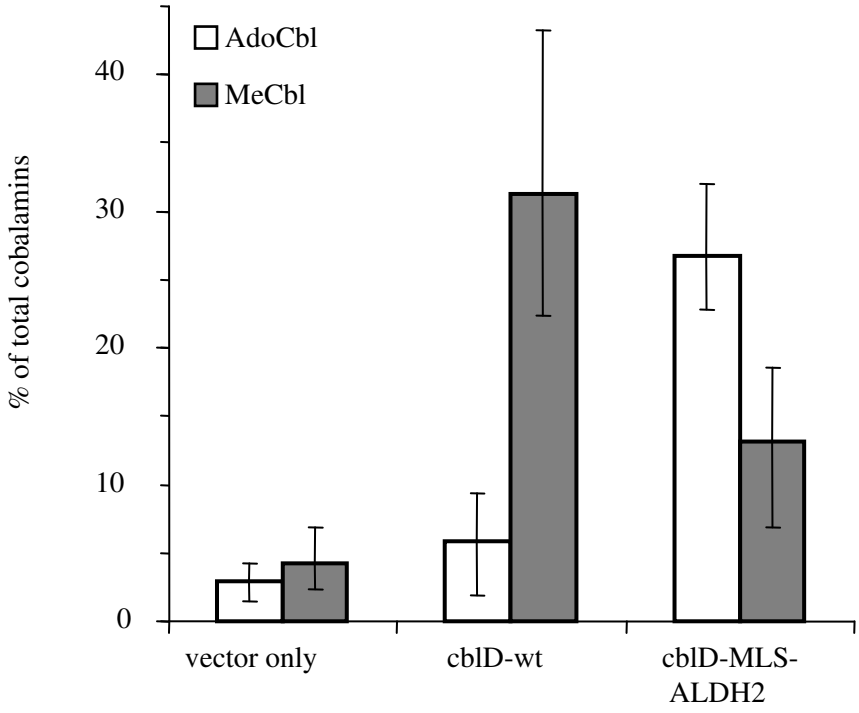
ALDH2: aldehyde dehydrogenase 2

MMADHC: methylmalonic aciduria, cblD type, and homocystinuria

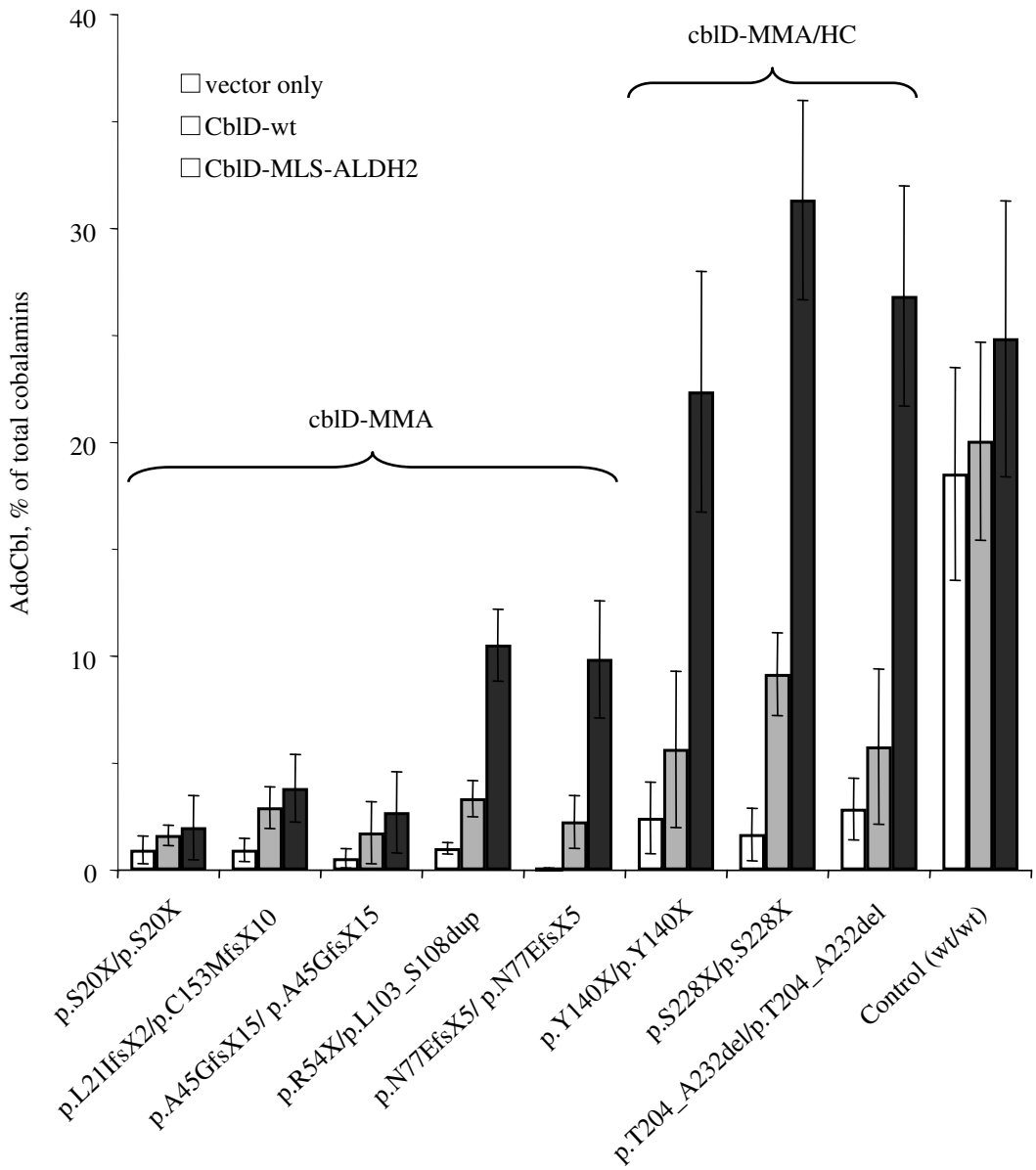
PTC: premature termination codon

NMD: nonsense mediated mRNA decay

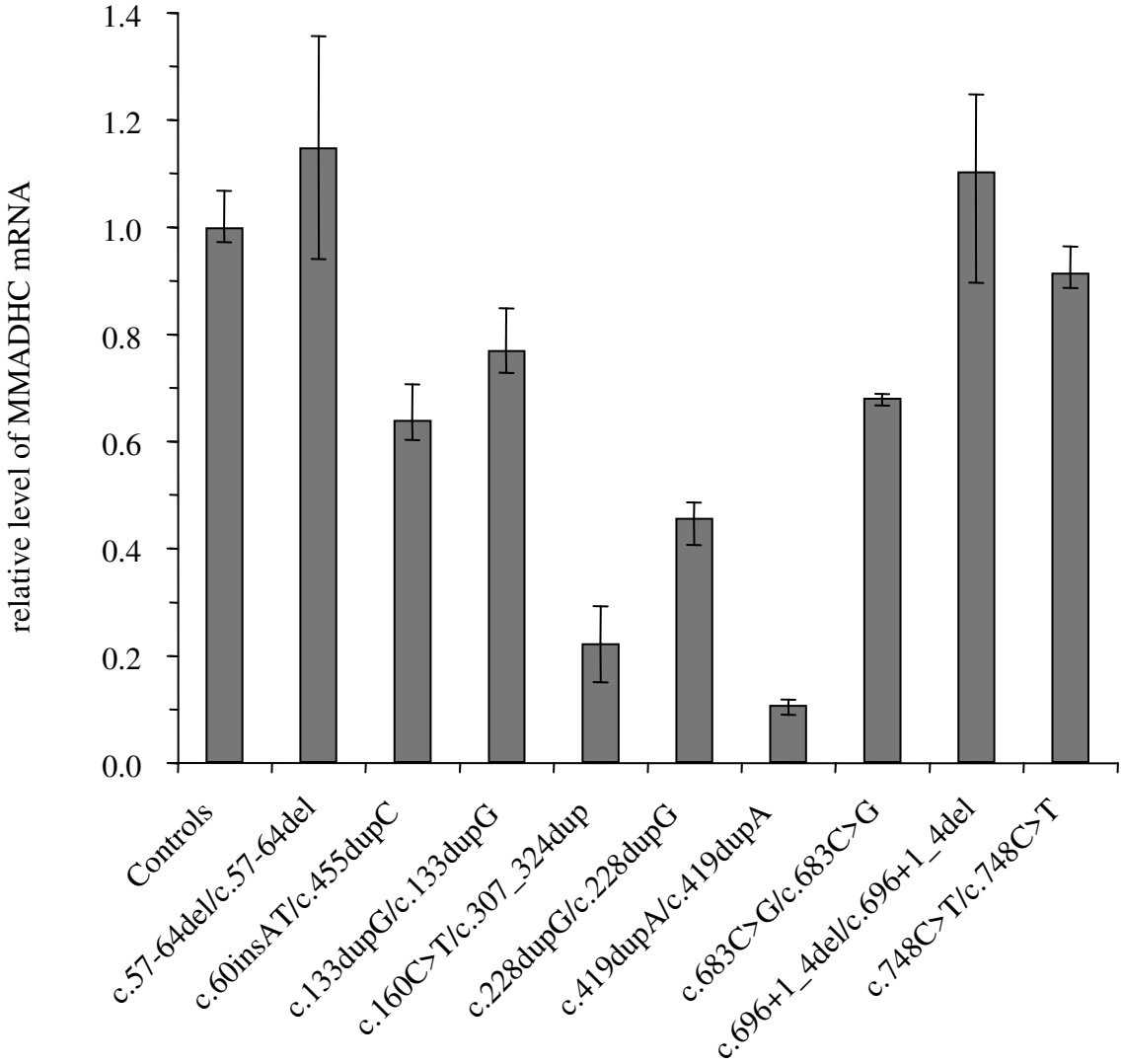
Stucki et al. Fig 1



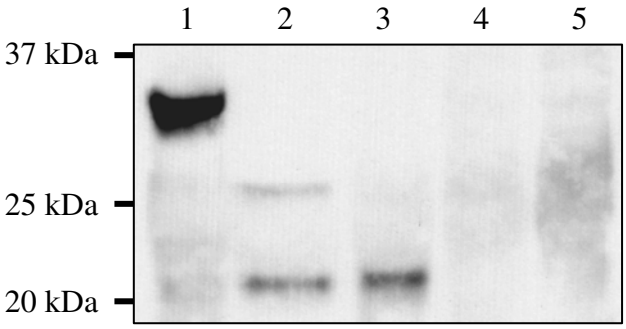
Stucki et al. Fig 2



Stucki et al. Fig 3a

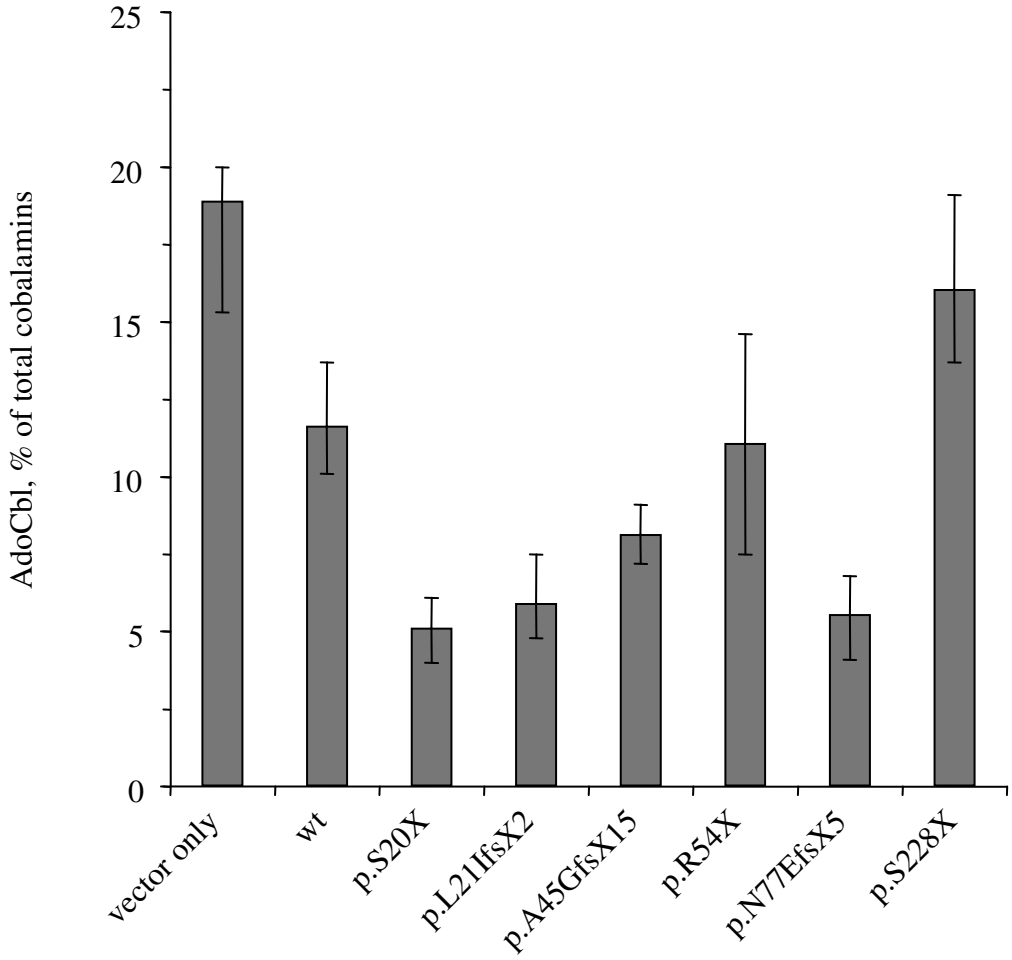


Stucki et al. Fig 3b

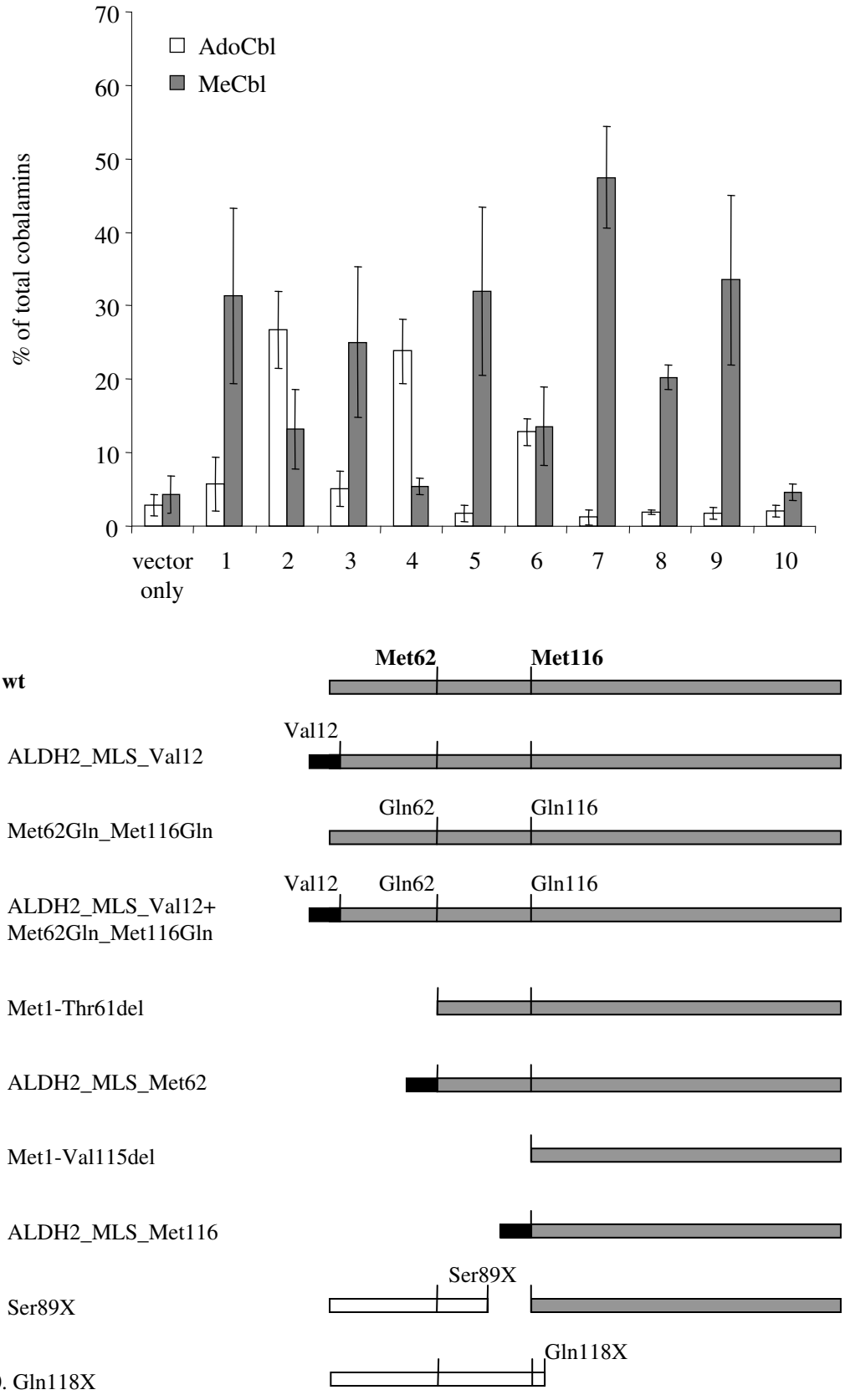


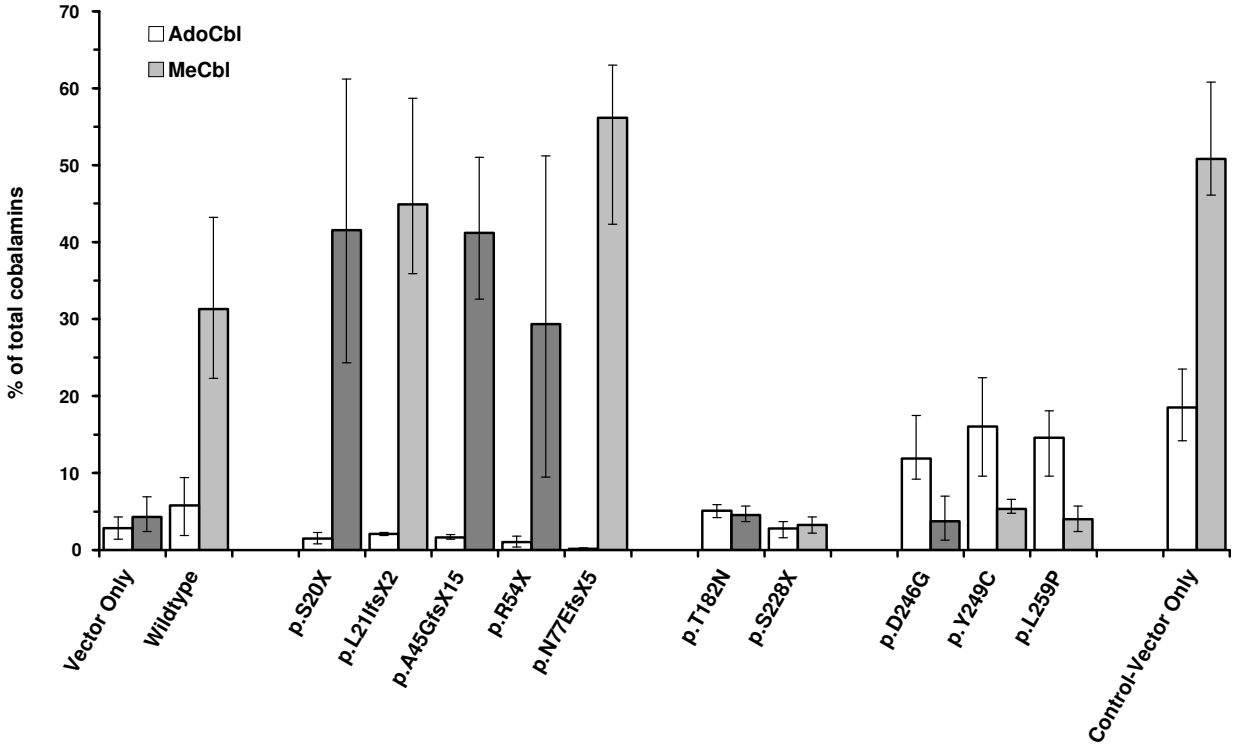
Cell line		**MMADHC allele**	
1.	wt		
2.	D003	c.57-64del	(p.Ser20X)
3.	D017	c.228dupG	(p.Asn77GlufsX5)
4.	D006	c.419dupA	(p.Tyr140X)
5.	D007	c.696+1_4_delGTGA	(p.Phe204_Ala232del)

Stucki et al. Fig 4

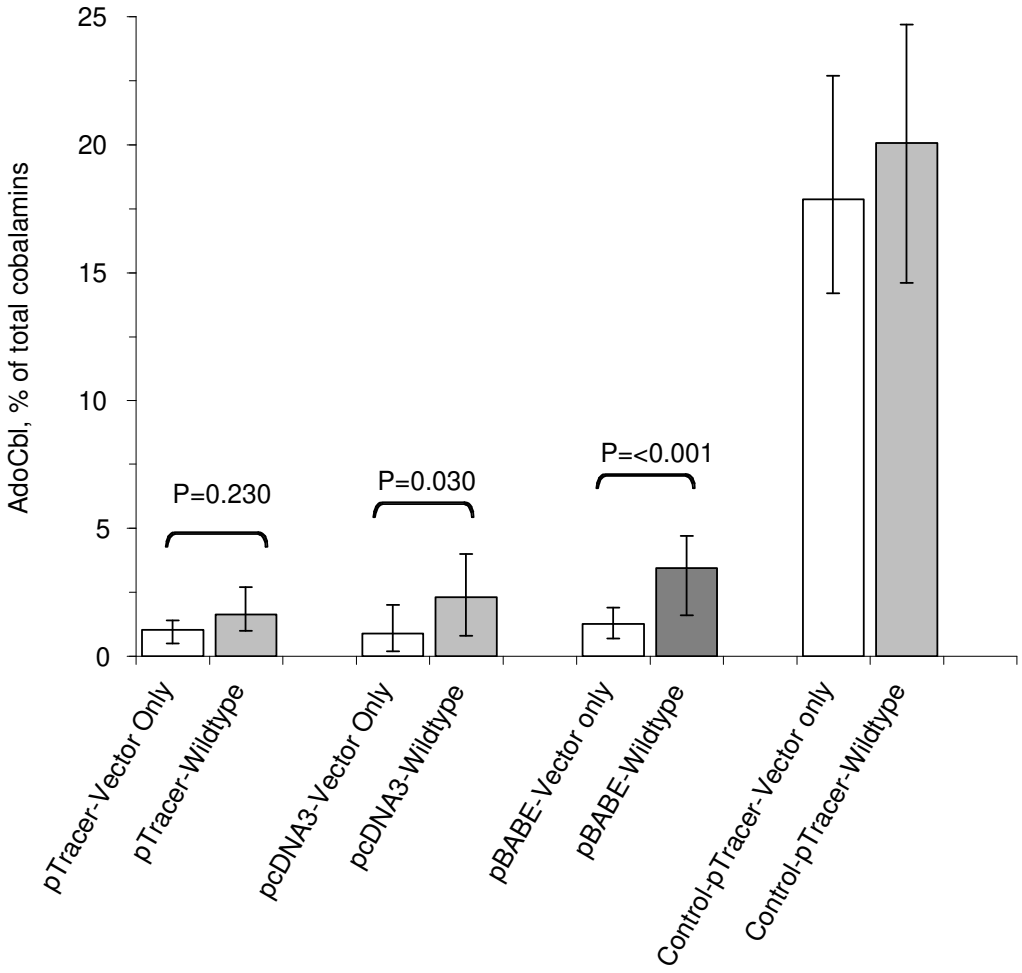


Stucki et al. Fig 5

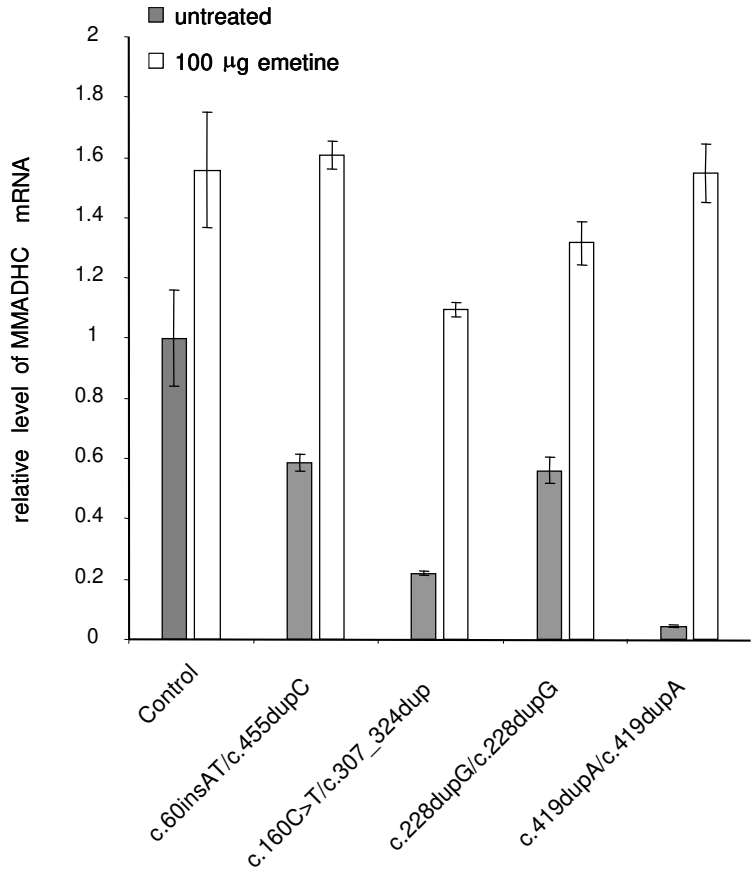




Supplementary Figure S1. **Expression of the wildtype (wt) and 10 mutant alleles detected in cblD patients in transformed fibroblasts of a cblD-MMA/HC patient (D007).** Five mutations (p.S20X, p.L21IfsX2, p.A45GfsX15, p.R54X, p.N77EfsX5) are associated with the cblD-MMA, three (p.D246G, p.Y249C, p.L259P) with the cblD-HC and one (p.S228X) with the cblD-MMA/HC phenotype. One mutation (p.T182N) was detected in the heterozygous state in a cblD-HC patient (D002) with the p.Y249C mutation on the second allele. Expression of five of the mutant alleles (p.S20X, p.R54X, p.T182N, p.Y249C, p.L259P) has been reported earlier (Coelho et al 2008 NEJM 358:1454-64). Transfections of the cblD-MMA/HC cell line and a transformed cell line of a healthy control with an empty vector (vector only) were used as controls. Constructs were prepared using pTracer vector, transfections were made by electroporation and rescue of function was detected by the determination of the synthesis of adenosylcobalamin (AdoCbl) and methylcobalamin (MeCbl) from [^{57}Co]cyanocobalamin. Columns represent the mean and vertical lines the range of results from at least 4 replicate experiments with single determinations.



Supplementary Figure S2. **Effect of the type of transfection vector and the method of transfection on rescue of adenosylcobalamin (AdoCbl) synthesis**. The *MMADHC* wildtype cDNA was transfected into transformed fibroblasts of a cblD-MMA patient homozygous for p.S20X. Transient transfection was performed by electroporation with constructs in the mammalian expression vectors pTracer and pcDNA3. For stable transfection in a retroviral system, constructs in pBABE vector were packed in virus particles using Lin-X packaging cells and used to infect cblD-MMA cells. For comparison AdoCbl synthesis in transformed fibroblasts of a healthy control after transfection with constructs in pTracer vector is shown. Transfections with empty vectors (vector only) were used as negative controls. Columns represent the mean and vertical lines the range of results from at least 4 replicate experiments with single determinations.



Supplementary Figure S3. **Inhibition of nonsense mediated decay in various patient and control cell lines.** Relative levels of *MMADHC* mRNA in fibroblasts of 3 cblD-MMA patients (D008, c.60insAT/c.455dupC; D004, c,160C>T/c.307_324dup; D017, c.228dupG/c.228dupG) and 1 cblD-MMA/HC patient (D006, c.419dupA/c.419dupA) in the presence (white columns) or absence (grey columns) of emetine. mRNA levels were compared to mean level obtained in 4 control cell lines and were normalized to mRNA level of GAPDH. Columns are the mean and vertical lines the range of triplicate determinations.

Supplementary Table 1. Primers used to produce *MMADHC* constructs

WT

Forward: gttctgggacagctggagac

Reverse: caatgtggatgtgttcaacg

ALDH2_MLS_Val12

Forward: gcctgggccgccgcctcttgtcagccgccgccacccaggccgtttcctatctcccaggat

Reverse: caatgtggatgtgttcaacg

Met62Gln

Forward: ggcctgatgaaactcagggacccttggacctcaag

Reverse: ccaaagggtccctgagtttcatcaggccacactgttc

Met116Gln

Forward: gtgaaagacatgagtttgtgcaggcacaatatgtg

Reverse: ctgaaattcattcacatattgtgcctgcacaaactcatg

Met1-Thr61del

Forward: acatagaattctgatgaaactatggga

Reverse: caatgtggatgtgttcaacg

Met1-Val115del

Forward: acatagaattcaagtgaaagacatga

Reverse: caatgtggatgtgttcaacg

Ser89X

Forward: ctcaatgggactgcttaacagaagaaaagcctggttc

Reverse: ggcttttcttctgttaagcagtcccattgaggtgacaatc

Gln118X

Forward: gttgtttgtgatggcataatatgtgaatgaatttcagggtaatgatg

Reverse: ttcattcacatattatgccatcacaaactcatgtctttcacttg